Arch Biol Sci. 2021;73(4):473-482 https://doi.org/10.2298/ABS210929041K

Effect of *PDYN* rs2281285, rs2235749 and rs910080 gene polymorphisms on the intensity of depression symptoms and negative craving in heroin addicts

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Received: September 29, 2021; **Revised**: October 14, 2021; **Accepted**: October 15, 2021; **Published online**: November 1, 2021

Abstract: The present study was undertaken to explore whether prodynorphin (*PDYN*) polymorphisms have an effect on the intensity of depressive symptoms and negative craving in heroin addicts in a sample of 100 heroin addicts and 108 controls. *PDYN* rs2281285, rs2225749 and rs910080 polymorphisms were analyzed by PCR-RFLP. Craving and the intensity of depressive symptoms were measured by the Substance Craving Scale and the Beck Depression Inventory-II, respectively. A significant association between depression severity and *PDYN* rs2281285 (P=0.026) and rs2225749 (P=0.038) polymorphisms was detected. *PDYN* rs2225749 variation showed a trend association with increased negative craving (P=0.066). We also examined the associations between heroin dependence and *PDYN* rs2281285, rs2225749 and rs910080 gene polymorphisms at the gene and haplotype levels. The AAA haplotype was more frequent in heroin addicts and shown to be significantly associated with increased risk for heroin dependence (OR, 8.922; 95% CI, 1.116-71.313; P<0.05). *PDYN* rs2281285 and rs2225749 variations affected the intensity of depressive symptoms, and *PDYN* rs2225749 polymorphism may contribute to the induction of negative craving in heroin addicts. Haplotype analysis revealed for the first time that addicts with the AAA haplotype of *PDYN* gene may be more prone to heroin dependence.

Keywords: heroin dependence; dynorphins; *PDYN* variation; depression; negative craving

INTRODUCTION

The endogenous opioid system of the brain tightly regulates nociception and emotional behaviors [1-3]. Additionally, it has been reported to be involved in psychiatric disorders characterized by reward dysfunction such as drug addiction and depression and other disorders of excessive consumption such as obesity [2,3]. There are 3 G protein-coupled opioid receptors: the mu-opioid receptor (MOR), delta (DOR) and kappa (KOR), which are activated by different endogenous ligands (β-endorphin, enkephalin, dynorphin, respectively) under physiological conditions [2,3]. The stimulation of MOR and DOR is highly reinforcing and euphoric. Conversely, the activation of KOR is generally aversive [3]. Among endogenous opioid peptides, dynorphins poorly interact with MOR or DOR and bind predominantly KORs, producing an unpleasant or aversive state in humans [3-5]. Thus, the dynorphin/KOR system exerts complex and opposing effects on reward-related behaviors in the opioid system [3,6].

The dynorphin/KOR system is composed of the dynorphin neuropeptide family and KORs [1,7]. This system is expressed throughout both central and peripheral nervous systems [8], producing an inhibitory effect on brain reward function by suppressing dopamine release from brain regions associated with the development of drug dependence, such as the mesolimbic reward pathway and the nigrostriatal pathway [9]. Dynorphins are a class of opioid peptides that are derived from the precursor protein prodynorphin (PDYN) [10]. When PDYN is cleaved mainly by proprotein convertase-2, multiple active peptides such as

How to cite this article: Kaya-Akyüzlü D, Özkan-Kotiloğlu S, Yalçın-Şahiner Ş, Ayaz N. Effect of *PDYN* rs2281285, rs2235749 and rs910080 gene polymorphisms on the intensity of depression symptoms and negative craving in heroin addicts. Arch Biol Sci. 2021;73(4):473-82.

dynorphin A, dynorphin B and α/β-neo-endorphin are released from the presynaptic terminal of depolarized PDYN-containing neurons [8,9]. Dynorphin is an integral part of the brain's stress response system stimulated during painful, noxious or stressful conditions [3]. Additionally, dynorphins have a crucial role in compulsive drug-seeking behavior, anxiety, anhedonia, dysphoria, which make up negative emotional states, a compelling motivation for relapse to most illicit substances [7,11,12].

Drug dependence is a vicious cycle consisting of binge/intoxication, withdrawal/negative effect, and preoccupation/anticipation stages. Escaping this recurring cycle is a real challenge for addicted people [9,13]. In the last stage of this cycle, craving leads to drug-seeking behavior resulting in relapse [13]. Opioid use disorder (OUD) is a major public health challenge worldwide [12,14,15]. It is a chronic, progressive and complex neurobiological disorder associated with changes in neuroadaptive mechanisms causing dependence, craving and relapse [10,12]. Craving and relapse are the main problems in the treatment of addiction [16]. Additionally, epidemiological studies have shown high comorbidity between opiate addiction and major depressive disorder due to overlapping neurobiological abnormalities in neural circuits regulating emotion [11]. It is considered that depressive symptoms such as social withdrawal may also cause relapse [13]. Although craving and depression are the main characteristics of the drug addiction, cumulative evidence proposes that these addictive symptoms may depend not only on environmental but also on genetic features. To date, there are a few studies showing the effect of *PDYN* gene polymorphisms, such as rs910080, rs1022563, rs1997794, rs35286281, on heroin dependence risk in individuals of different ethnicity [17-22]. To the best of our knowledge, none of these studies have examined the effects of *PDYN* gene polymorphisms on negative craving and depressive symptoms in heroin addicts. Based on this background, we hypothesized that *PDYN* gene polymorphisms (rs2281285, rs2225749 and rs910080) affect the level of depressive symptoms (reflected by an elevated Beck Depression Inventory-II score) and negative craving (reflected by an elevated Substance Craving Scale score) in heroin addicts. To test this hypothesis, we evaluated the association of each phenotype with individual single nucleotide polymorphisms (SNPs). Furthermore, our second goal was to examine the effect of *PDYN* polymorphisms and haplotypes containing the *PDYN* rs910080, rs2281285 and rs2225749 SNPs on heroin dependence risk.

MATERIALS AND METHODS

Study population

A total of 100 heroin addicts and 108 healthy controls were enrolled in this study. All heroin addicts were recorded in the Ankara Training and Research Hospital (AMATEM Clinic) in Ankara, Turkey. They were diagnosed with heroin dependence and fulfilled the criteria for opioid use disorder according to the Diagnostic and Statistical Manual of Mental Disorders (DSM–5) diagnostic criteria. The exclusion criteria for study subjects were as follows: (i) clinically significant comorbid psychiatric illness such as any psychotic disorders, schizophrenia, mental retardation, bipolar disorder and severe depression; (ii) substance use disorders other than heroin and nicotine dependence. Controls were comprised of healthy individuals that satisfied the same exclusion criteria and had no past or current substance use disorder (n=108). Controls were matched to heroin addicts by gender and smoking habits. Subjects identifying themselves as Turkish were included in the study. Written informed consent and permission to use their information for future studies of heroin dependence and related phenotypes was obtained from each participant who was eligible for the study. A small questionnaire was used to gather sociodemographic information regarding age, marital, education and employment status, past and present substance use, duration of heroin use, age at onset of dependence, quantity of heroin consumed (g/day). The study design was approved by the institutional ethics committee (Approval No: I4-207-20 in 2020). Samplings were performed in accordance with the principles of the Declaration of Helsinki.

Determination of the *PDYN* rs2281285, rs2225749 and rs910080 polymorphisms

Two mL of venous blood was taken from each individual into tubes with ethylenediaminetetraacetic acid (EDTA) for DNA isolation, and the tubes were kept at -20°C. Genomic DNA was extracted from whole-blood

samples using the QIAamp DNA blood-kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Genotyping was carried out using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP), as previously described [20]. PCR amplification was conducted on a Techne Tc 512 PCR System in a 50-μL reaction mixture containing 200 μM of dNTPs, 10 pmol each of the forward (F) and reverse (R) primers, 1 U of Hot Star Taq DNA polymerase (Qiagen, US), 10×PCR buffer (Qiagen) and 50 ng of genomic DNA. Following confirmation of amplification by gel electrophoresis, the PCR products were digested in a reaction containing 5 U of restriction enzyme. Primer sequences, PCR conditions, restriction enzymes, and restriction fragment lengths are given in Supplementary Table S1. The undigested and digested PCR products were separated by gel electrophoresis on a 3% agarose gel, visualized by ethidium bromide staining under an UV illuminator, and then scanned and photographed using the Syngene Monitoring System (Supplementary Figs. S1-S3). All results of RFLP for each variant in 30 randomly selected samples were confirmed by DNA sequencing using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (ThermoFisher Scientific) on an ABI Prism 3100 Genetic Analyzer. The automated DNA sequencing was employed to confirm the authenticity of the amplified PCR products.

Measurements

To investigate the effects of *PDYN* polymorphisms on the intensity of depressive symptoms and craving, heroin addicts were assessed using the Substance Craving Scale (SCS) and Beck Depression Inventory-II (BDI-II). The SCS is the version of the Penn Alcohol Craving Scale (PACS) used to evaluate the craving for substances other than alcohol. The PACS was developed to evaluate the desire for alcohol for the previous week. The SCS is a 5-item self-reporting measure scored between 0 and 6. The questions are about the frequency, intensity and duration of craving, with a demonstrated validity and reliability of a Turkish version of the PACS [23]. BDI-II is a Likert-type 21-item self-reporting tool developed to measure characteristic attitudes and symptoms of depression such as mood, sense of failure, guilt, punishment, suicidal ideas, irritability, social withdrawal, indecisiveness, work difficulty, fatigability and somatic preoccupation in normal and psychiatric populations. This scale ranges from 0 to 3 and the total score is calculated by the sum of item scores (ranging from 0 to 63). The validity and reliability of a Turkish version of the BDI-II was demonstrated [24].

Statistical analyses

The Statistical Package for Social Sciences ver. 21.0 software for Windows was used for statistical analyses. The frequencies of the *PDYN* rs2281285, rs2225749 and rs910080 alleles and genotypes were obtained by direct counting, and departure from the Hardy-Weinberg equilibrium was evaluated by the chi-square test. In the exploratory analysis, data showed a normal distribution (according to the Kolmogorov-Smirnov test), parametric Student's t-test or one-way ANOVA were used to compare 2 or 3 independent groups in terms of metric variables. For data showing non-normal distribution, the nonparametric Mann-Whitney U test or the Kruskal-Wallis test were used. Data are presented as the mean and standard deviation (SD) or median and the interquartile range (IQR) according to the normality of the data. For categorical data, numbers and percentages are given. The relationship between the *PDYN* polymorphisms and heroin dependence was modeled by binary logistic regression analysis. Odds ratio (OR) value and 95% confidence intervals were calculated for comparing the risk of dependence, and the wild-type genotype served as a reference group. The relationship between BDI-II and SCS scores was analyzed using Pearson's correlation test. The effects of all 3 SNPs were also determined on the allelic level via haplotype analysis. Linkage disequilibrium (LD) analysis and haplotype-based case-control analysis were performed using SHEsis software to evaluate the combined effect of both polymorphisms [25,26]. Haplotypes with frequencies <3% in both cases and controls were omitted. Linkage disequilibrium was followed by the D' statistic, and a D' value of ≥0.8 indicated that related SNPs formed one block [25]. $P<0.05$ was considered as statistically significant.

RESULTS

There was a total of 100 heroin addicts in the cohort, 91 of which were males (91.0%) and 9 were females (9.0%), and of the 108 healthy controls, 98 were males

Table 1. Genotype frequencies of *PDYN* rs910080, rs2281285 and rs2235749 polymorphisms in heroin addicts and healthy subjects.

***PDYN* rs910080 Genotypes**	**Heroin Addicts**		**Healthy controls**		**P-value**	**Odds Ratio (95% CI)**
	n	**%**	**n**	**%**		
AA	49	49	48	44.4	P>0.05	Reference
AG	36	36	53	49.1		2.09 (0.787-5.601)
GG	15	15	7	6.5		3.155 (1.170-8.507)
Variant allele freq.	33%		31%			
HWE p-value	$\chi2$=3.46; P=0.06		$\chi2$=2.33; P=0.13			
***PDYN* rs2281285 Genotypes**	**Heroin Addicts**		**Healthy controls**		**P-value**	**Odds Ratio (95% CI)**
	n	**%**	**n**	**%**		
AA	74	74	76	70.4	P>0.05	Reference
AG	20	20	27	25.0		0.834 (0.454-1.533)
GG	6	6	5	4.6		
Variant allele freq.	16%		17%			
HWE p-value	$\chi2$=6.55; P=0.01		$\chi2$=1.54; P=0.21			
***PDYN* rs2235749 Genotypes**	**Heroin Addicts**		**Healthy controls**		**P-value**	**Odds Ratio (95% CI)**
	n	**%**	**n**	**%**		
GG	45	45	50	46.3	P>0.05	Reference
GA	38	38	50	46.3		0.424 (0.167-1.075)
AA	17	17	8	7.4		1.184 (0.661-2.122)
Variant allele freq.	36%		31%			
HWE P-value	$\chi2$=3.07; P=0.08		$\chi2$=0.89; P=0.34			

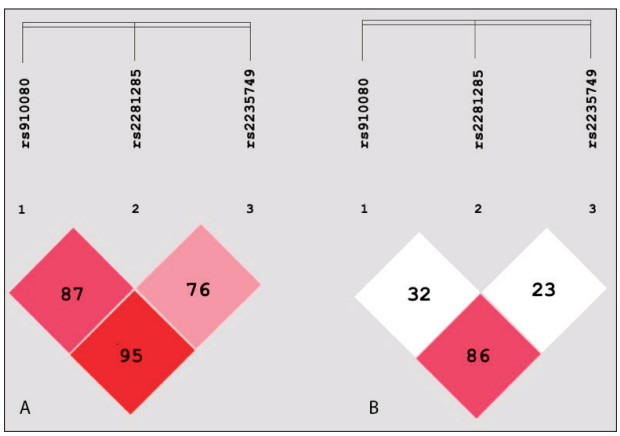

Fig. 1. (**A**) Linkage disequilibrium (D') and (**B**) correlation coefficient (r2) of 3 studied gene variants.

and 10 females (about 91.0% and 9.0%, respectively). The characteristics of the 108 healthy controls and 100 addicts are presented on Supplementary Table S2. Additionally, the clinical features of heroin addicts are shown in Supplementary Table S3.

The genotype and allele frequencies of *PDYN* rs910080, rs2281285 and rs2235749 polymorphisms in heroin addicts (n=100) and controls (n=108) are presented in Table 1. For addicts, the genotype and allele frequencies of *PDYN* rs910080 and rs2235749 polymorphisms were consistent with Hardy-Weinberg equilibrium ($\chi2$=3.46, P=0.06; $\chi2$=3.07, P=0.08, respectively), but those of *PDYN* rs2281285 were not ($\chi2$=6.55, P=0.01). As for the controls, the genotype and allele frequencies of all 3 polymorphisms were consistent with Hardy-Weinberg equilibrium (P>0.05). The relationship between the *PDYN* genotypes and heroin addiction was examined by logistic regression analysis and none of the *PDYN* polymorphisms were found to be associated with heroin dependence (Table 1).

Fig. 1 shows the patterns of LD in the *PDYN* gene, with their |D'| and r2 values. The LD test for all pairs of markers in *PDYN* showed strong LD (r^2>0.8) for rs910080 and rs2235749. Our results indicate that these 2 SNPs were in one LD block with a defined haplotype frequency. The A-A-A haplotype, which was more frequent in heroin addicts, was significantly associated with increased risk for heroin addiction (OR, 8.922; 95% CI, 1.116-71.313; P<0.05). However, the haplotypes A-A-G (OR, 0.784; 95% CI, 0.520-1.184; P>0.05), G-A-A (OR, 1.202; 95% CI, 0.718-2.014; P>0.05), G-G-A (OR, 0.936; 95% CI, 0.538-1.626; P>0.05) showed no significant association with the risk for heroin dependence (Table 2). The frequencies of rs910080-rs2235749, rs910080-rs2281285 and rs2235749-rs2281285 haplotypes were analyzed and only the A-A haplotype of *PDYN* rs910080-rs2235749 was found to be more frequent in heroin addicts as compared to controls (OR, 8.961; 95% CI, 1.122-71.533; P >0.05). None of the other haplotypes showed significance with the risk for heroin dependence.

The BDI-II scores of heroin addicts and controls according to heroin dependence and *PDYN* rs910080, rs2281285 and rs2235749 genotypes were compared

Table 2. Overall haplotype associations of SNPs according to SHEsis software.

Haplotype			Heroin addicts N (freq)	Controls N (freq)	Chi2	Fisher's P	Pearson's P	Odds ratio (95% CI)
rs910080	rs2281285	rs2235749						
A	**A**	**A***	8.07 (0.040)	1.01 (0.005)	6.160	**0.013098**	**0.013086**	8.922 (1.116-71.313)
A	**A**	**G**	123.59 (0.618)	144.42 (0.669)	1.340	0.247100	0.247025	0.784 (0.520-1.184)
G	**A**	**A**	36.34 (0.182)	33.57 (0.155)	0.491	0.483466	0.483448	1.202 (0.718-2.014)
G	**G**	**A**	27.59 (0.138)	31.42 (0.145)	0.056	0.813442	0.813425	0.936 (0.538-1.626)
A	**A**	-	131.68 (0.658)	145.42 (0.673)	0.159	0.689975	0.689974	0.919 (0.608-1.390)
G	**A**	-	36.32 (0.182)	33.58 (0.155)	0.476	0.490455	0.490439	1.199 (0.716-2.006)
G	**G**	-	29.68 (0.148)	33.42 (0.155)	0.040	0.840594	0.840573	0.946 (0.553-1.620)
A	-	**A***	8.07 (0.04)	1.01 (0.005)	6.201	**0.012797**	**0.012785**	8.961 (1.122-71.533)
A	-	**G**	125.93 (0.630)	147.99 (0.685)	1.414	0.234506	0.234429	0.780 (0.518-1.175)
G	-	**A**	63.93 (0.320)	64.99 (0.301)	0.178	0.673345	0.673346	1.094 (0.721-1.659)
-	**A**	**A**	44.86 (0.224)	34.99 (0.162)	2.559	0.109708	0.109641	1.493 (0.912-2.444)
-	**A**	**G**	123.14 (0.616)	144.01 (0.667)	1.347	0.245810	0.245734	0.784 (0.519-1.183)
-	**G**	**A**	27.14 (0.136)	31.01 (0.144)	0.061	0.805602	0.805587	0.933 (0.535-1.627)

(Table 3). It was found that the median BDI-II scores of heroin addicts (25.50; IQR:15.0-34.75) were significantly higher than those of controls (5.0; IQR:1.0-10.0) (z=-9.832; P<0.001). When BDI-II scores of *PDYN* rs910080 genotypes were compared, the BDI-II scores of the AA genotype (24.18±14.70) were lower than those of the AG (26.11±13.40), GG (27.67±8.28) and AG+GG (26.56±12.06) genotypes; however these differences were not statistically significant. As for the *PDYN* rs2281285 polymorphism, heroin addicts with the GG genotype merged with those with AG genotype due to low frequency and were compared statistically to those with the AA genotype by the independent sample t-test. The BDI-II score of the *PDYN* rs2281285 AA genotype was significantly lower (24.27±14.31) than that of the AG+GG genotypes (28.61±9.94) (t=-1.429, P=0.026). Comparison of the BDI-II total of *PDYN* rs2235749 GG, GA and AA genotypes showed that there was no statistically significant difference between genotypes (F=0.471, P=0.626), although heroin addicts with GG genotype (24.11±15.21) had lower BDI-II total scores than those with GA (25.92±12.87) and AA (27.64±9.04) genotypes. To examine the effect of the *PDYN* rs2235749 A allele on the BDI-II total scores, GA and AA genotypes were merged and compared to GG genotypes, revealing that heroin addicts with the A allele (26.45±11.76) had significantly higher BDI-II scores than those with the G allele (24.11±15.21) (t=-0.869; P=0.038).

The mean SCS score of heroin addicts was 17.32±8.58 with a minimum of zero and a maximum of 32. The SCS scores were also compared according to the *PDYN* genotypes (Table 3). Although the mean SCS scores were not statistically different (t=-1.232; P=0.112), the mean SCS scores of the *PDYN* rs910080 and rs2281285 AA genotypes were lower (16.24±9.46 and 16.31±8.98, respectively) than those of the AG+GG genotypes (18.35±7.58 and 20.19±6.62, respectively). Unlike *PDYN* rs910080 and rs2281285 polymorphisms, the mean scores of the *PDYN* rs2235749 GG genotype (16.53±9.57) were significantly lower than those of the GA+AA genotypes (17.96±7.70) (t=-0.828; P=0.066). In addition, Pearson's correlation test revealed that there were significant and positive correlations between the SCS score and the scores of BDI-II, the Clinical Opiate Withdrawal Scale (COWS) and the Beck Anxiety Inventory (BAI) (r=+0.514, P<0.001; r=+0.341, P<0.001; r=+0.379, P<0.001, respectively), showing that craving in heroin addicts was negatively reinforced.

Some clinical characteristics of heroin addicts according to the *PDYN* genotypes are shown in Table 4. None of the parameters in this table were normally distributed, thus, median values of genotypes were compared using the Mann-Whitney U or Kruskal-Wallis tests. Heroin addicts with *PDYN* rs910080 AG+GG genotypes ($\tilde{x}$=7 years; IQR:5.0-18.0) had a

Table 3. BDI-II and SCS total scores of heroin addicts according to *PDYN* rs910080, rs2281285 and rs2235749 genotypes.

***PDYN* rs910080 Genotypes**	**BDI-II Total scores**		**P-value**	**SCS total scores**		**P value**
	Mean±SD	Min.-Max.		Mean±SD	Min.-Max.	
AA (n=49)	24.18±14.70	0.0-63.0	F=0.462 P=0.632	16.24±9.46	0.0-32.0	F=0.751 P=0.474
AG (n=36)	26.11±13.40	0.0-58.0		18.33±7.83	0.0-32.0	
GG (n=15)	27.67±8.28	14.0-51.0		18.40±7.21	8.0-32.0	
AG+GG (n=51)	26.56±12.06	0.0-58.0	t=-0.888 P=0.095	18.35±7.58	0.0-32.0	t=-1.232 P=0.112
***PDYN* rs2281285 Genotypes**						
AA (n=74)	24.27±14.31	0.0-63.0	t=-1.429 **P=0.026**	16.31±8.98	0.0-32.0	t=-2.016 P=0.154
AG+GG (n=26)	28.61±9.95	11.0-51.0		20.19±6.62	8.0-32.0	
***PDYN* rs2235749 Genotypes**						
GG (n=45)	24.11±15.21	0.0-63.0	F=0.471 P=0.626	16.53±9.57	0.0-32.0	F=0.340 P=0.713
GA (n=38)	25.92±12.87	0.0-58.0		17.97±7.79	0.0-32.0	
AA (n=17)	27.65±9.04	13.0-51.0		17.94±7.73	0.0-32.0	
GA+AA (n=55)	26.45±11.76	0.0-58.0	t=-0.869 **P=0.038**	17.96±7.70	0.0-32.0	t=-0.828 **P=0.066**
Total Median (IQR)	25.40±13.40 25.50 (15.0-34.75)	0.0-63.0	Z=-9.832 **P=0.001**	17.32±8.58	0.0-32.0	-
Controls Median (IQR)	6.70±6.84 5 (1.0-10.0)	0.0-32.00		-	-	

Table 4. Comparison of heroin addicts according to the *PDYN* rs910080, rs2281285 and rs2235749 genotypes in view of heroin addiction and heroin use.

***PDYN* rs910080 Genotypes**	The onset age of heroin (years)	Heroin addiction (years)	The daily amount of heroin consumed (g/day)	Without heroin's longest time of abstinence (months)
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
AA (n=49)	21.0 (17.5-23.0)	6.0 (3.0-8.0)	3.0 (1.0-5.0)	6.0 (1.0-16.5)
AG (n=36)	20.0 (17.25-23.75)	7.0 (5.0-10.0)	2.0 (1.13-3.0)	6.0 (1.0-12.0)
GG (n=15)	23.0 (20.0-24.0)	6.00 (3.0-6.0)	2.0 (1.0-3.0)	8.0 (2.0-18.0)
AG+GG (n=51)	21.0 (19.0-24.0)	7.0 (5.0-18.0)	2.0 (1.0-3.0)	6.0 (1.0-12.0)
P-value	0.164	**0.028**	0.285	0.763
***PDYN* rs2281285 Genotypes**				
AA (n=74)	21.0 (18.0-23.25)	6.75 (4.0-8.0)	2.25 (1.0-5.0)	7.0 (1.0-12.5)
AG+GG (n=26)	21.0 (18.75-23.25)	6.5 (4.75-8.0)	2.0 (1.0-3.25)	4.0 (1.0-12.0)
P-value	0.795	0.934	0.665	0.353
***PDYN* rs2235749 Genotypes**				
GG (n=45)	21.0 (18.0-23.0)	6.0 (3.0-8.0)	2.0 (1.0-5.0)	6.0 (1.0-13.5)
GA (n=38)	20.5 (16.75-24.25)	7.0 (4.88-8.5)	2.0 (1.0-3.0)	6.0 (1.0-12.0)
AA (n=17)	21.0 (19.0-23.5)	6.00 (5.0-7.0)	3 (1.0-4.0)	8.0 (1.5-24.0)
GA+AA (n=55)	21.0 (18.0-24.0)	7.0 (5.0-8.0)	2.0 (1.0-3.0)	7.0 (1.0-12.0)
P-value	0.082	0.830	0.669	0.112

heroin addiction that was of a statistically significantly longer duration than those with the AA genotype (x̃=6 years; IQR:3.0-8.0) (P=0.028). Although the median daily amount of heroin consumed per day was higher in heroin addicts with *PDYN* rs910080 AA genotypes (x̃=3 g/day; IQR: 1.0-5.0) than in those with AG+GG genotypes (x̃=2 g/day; IQR: 1.0-3.0), this difference was not statistically significant (P=0.285). Analysis of the heroin addicts according to the *PDYN* gene rs2281285 and rs2235749 genotypes revealed no differences between individuals with the homozygote wild-type genotype and those with heterozygote and homozygote mutant genotypes in view of age of onset, duration in years of heroin addiction, daily amount of heroin consumed (g/day) and the longest time of abstinence (in months).

DISCUSSION

The present study was undertaken with the aim of exploring whether *PDYN* gene polymorphisms (rs2281285, rs2225749 and rs910080) have an effect on the intensity of depressive symptoms and negative craving in heroin addicts due to the contribution of dynorphin to negative emotional states and to drug-seeking behavior as a result of negative reinforcement. In the literature, the contribution of the *PDYN* gene to the induction of negative craving [27-29] and depression [27] was investigated in only alcohol-dependent subjects. A few studies have examined the effects of *PDYN* genotypes on susceptibility to opiate addiction in different populations [17-22,30]. However, to the best of our knowledge, this is the first report examining the association of variations in the *PDYN* gene with negatively reinforced craving (anxiety and opiate withdrawal signs or symptoms) and the intensity of depressive symptoms at the gene level in heroin addicts.

Craving, an increased desire to use drugs, is associated with the use of substances that have euphoric properties and can be triggered by environmental factors. It is suggested that craving may arise from the desire for the rewarding features of opioids (positive craving) or the desire for drinking in the context of alleviating withdrawal symptoms (negative craving) [28]. The heroin addicts examined in the present study were characterized as possessing negative craving because of a positive significant correlation between craving and depression (according to the BDI-II scale), withdrawal symptoms (COWS scale) and anxiety (BAI scale) ($r=+0.514$, $P<0.001$; $r=+0341$, $P<0.001$; $r=+0.379$, $P<0.001$, respectively; data not shown). In the current analysis, although it was not statistically significant ($P=0.154$), the *PDYN* rs2281285 AG+GG genotypes (20.19±6.62) had a higher SCS total score than AA genotypes (16.31±8.98), suggesting the association of opiate negative craving with the *PDYN* rs2281285 variant. This is consistent with previous investigations on alcohol-dependent subjects, which demonstrated an association between the rs2281285 minor G allele and increased negative craving [27-29].

In the present study, a statistically significant association of the minor allele of the *PDYN* rs2281285 variant with the intensity of depressive symptoms in heroin addicts ($P=0.026$) was revealed. On admission, the BDI-II scores of AG+GG genotypes (28.61±9.95) were higher than those of the AA genotype (24.27±14.31). A trend for the association of BDI-II with the *PDYN* gene haplotype (rs2281285-rs1997794), but not for individual *PDYN* rs2281285, was reported in alcohol-dependent subjects [27]. To the best of our knowledge, there is no study examining the effect of *PDYN* rs2281285 genotypes on depressive symptoms in substance addicts. At the gene level, the inconsistent findings between our study and [27] suggested that the effect of this polymorphism may be dependent on ethnicity and/or consumed drugs. The *PDYN* rs2281285 polymorphism is located in the second intron (-749 bp downstream of the exon 2/intron B border). This area is thought to be involved in the regulation of transcription initiation from multiple sites, giving rise to 1 of 2 dominant transcripts: FL2-PDYN (in the hypothalamus and claustrum) and FL1-PDYN (in limbic-related structures) mRNAs [31]. Differential regulation of these 2 transcripts may occur due to allelic variability in this location [27]. The low risk rs2281285 A allele resides within the sequence that represents the DNA-binding element for several transcription factors of the POU (Pit-Oct-Unc) family. However, the high-risk rs2281285 minor G allele disables this sequence [27,28]. It was hypothesized that dynorphins expressed in the hypothalamus and pituitary gland, in which members of the POU family of transcription factors are involved in the regulation of gene expression, could have an important role in hormonal stress responses [27]. Consistent with this hypothesis, in the present study heroin addicts with a minor G allele exhibited more severe depression and a greater desire to drink in order to avoid the unwanted symptoms of withdrawal and mood dysregulation. However, more research is necessary to determine the functional importance of the *PDYN* rs2281285 variant.

We also found a significant association between *PDYN* rs2235749 and the intensity of depression symptoms ($P=0.038$). Heroin addicts with rs2235749 GA+AA genotypes (26.45±11.76) had a higher mean BDI-II score than those with the GG genotype (24.11±15.21). Similarly, on admission, the SCS scores of GA+AA genotypes (17.96±7.70) were higher than that of the GG genotype (16.53±9.57) ($P=0.06$). G/G homozygous individuals possessed a decreased novelty-seeking trait, a significant contributor to the development of illicit substance use, as compared to other groups [32]; they also demonstrated that rs2235749

polymorphism in the *PDYN* 3'-untranslated region (3'UTR) modifies striatal *PDYN* mRNA expression via impaired binding of miR-365, a microRNA that targets the 3'UTR of *PDYN*. Furthermore, novelty seeking, reward and motivated behavior are directly related to the manipulation of miR-365 activity in Pdyn-expressing neurons in the medial nucleus accumbens shell [32]; as previously suggested, we also propose that the decreased craving in rs2235749 G/G homozygous individuals compared with GA+AA genotypes may be related to the decreased novelty-seeking trait due to impaired binding of miR-365 to the *PDYN* 3'UTR-dependent rs2235749 allele. In addition, high novelty seeking may increase depressive symptoms in heroin addicts with GA+AA genotypes as compared to G/G homozygous individuals. Our hypothesis is consistent with a previous study that reported that novelty seeking apparently contributed to the frequency of depression [33].

PDYN rs910080 is located in the 3'-UTR region of the *PDYN* gene and is in high linkage disequilibrium with rs2235749 [32]. There was no significant association between *PDYN* rs910080 and the intensity of depressive symptoms (P=0.632) and negative craving (P=0.474). However, heroin addicts with the rs910080 AA genotype (24.18±14.70) had a lower mean BDI-II score than those with AG (26.11±13.40) and GG genotypes (27.67±8.28). In addition, the SCS score of AA genotype (16.24±9.46) was lower than those of AG+GG genotypes (18.35±7.58). It was hypothesized that rs910080 (AA/AG vs GG) is responsible for an increase in dynorphin peptides and associated with worse treatment outcome [34]. Our findings showing that *PDYN* rs910080 GG homozygous addicts had more intense depressive symptoms and increased heroin craving were not in agreement with Randesi's hypothesis [34]. Furthermore, the Mann-Whitney test detected significantly longer duration of heroin addiction in addicts with *PDYN* rs910080 AG+GG ($\tilde{x}$=7 years; IQR:5.0-18.0) compared to the AA genotype ($\tilde{x}$=6 years; IQR:3.0-8.0) (P=0.028). This finding suggested that rs910080 AG+GG genotypes may be a risk factor for drug-seeking behavior in heroin addicts due to the increased novelty seeking with the same mechanism as previously suggested [32].

Dynorphin, which is expressed throughout limbic brain areas, is considered an integral part of the brain's stress response system, and release of dynorphin occurs during exposure to painful, noxious or stressful stimuli [3,4]. Opioids are also strong activators of the brain stress system [35]. We also examined the association between heroin dependence and *PDYN* gene polymorphisms. Our findings show that rs910080 A>G, rs2235749 G>A and rs2281285 A>G variants of the *PDYN* gene are not associated with heroin dependence (OR=0.914, 95%CI=0.607-1.377, P>0.05; OR=0.790, 95%CI=0.529-1.181, P>0.05; OR=1.072, 95%CI=0.666-1.725, P>0.05, respectively) in Turkish individuals. To date, the association between *PDYN* rs910080 polymorphism and heroin dependence in Iranian [20], Asian [21] and Han Chinese [18] populations, and female European-Americans (EA), but not in the total EA population, has been shown [19]. As regards rs2235749 polymorphism, our results are consistent with [20,21] where it was shown that this polymorphism was not correlated with heroin dependence [20,21]. On the other hand, a significant pointwise correlation of the rs2235749 variant with heroin dependence was found [18]. Until now, the possible association between rs2281285 variants with the risk of heroin addiction was examined [20], and as in our findings, the authors reported no association between *PDYN* rs2281285 and heroin dependence. Inconsistent results from our and previous studies across different populations indicate that the *PDYN* gene appears to be associated with the risk of heroin dependence. This inconsistency could be due to several factors, including sample size and ethnic heterogeneity. It has been established that the products of the *PDYN* gene play roles in reward, mood regulation, motor functions and stress responses and that *PDYN* mRNA is found in brain regions associated with drug-withdrawal and drug-seeking [36,37]. Thus, studies with larger sample sizes examining different populations are needed to guarantee a robust and convincing conclusion.

We further investigated the interaction between rs2235749 and rs910080 in 3'UTR and rs2281825 in the second intron and observed a strong linkage disequilibrium between rs2235749 and rs910080 in 3'UTR, which is consistent with previous studies [18]. Although single SNP associations were not observed between rs2281285, rs1997794 and rs910080 SNPs in heroin dependence, haplotype analysis revealed that significantly more AAA haplotypes were found in heroin addicts than in controls. These results indicated for the first time that people with the AAA haplotype of the *PDYN* gene may be more prone to

heroin dependence. A candidate haplotype containing the *PDYN* rs2235749-rs910080 SNPs that were previously associated with alcohol dependence was herein also associated with heroin dependence (P=0.012785). Furthermore, the haplotype of the latter 2 SNPs was shown to be associated with low pre-existing *PDYN* expression [22].

The relationship between addiction and depression is undoubtedly bidirectional and comorbidity of depression and addiction is very frequent. Repeated exposure to illicit substances triggers neuroadaptations in brain structures and leads to depressive disorders. Conversely, depressed individuals may take advantage of the acute euphoric properties of illicit substances to overcome their depressed mood (the self-medication hypothesis). This hypothesis is convincingly supported [38]. Our findings show that heroin addicts (25.50; IQR:15.0-34.75) have a significantly higher median BDI-II score than healthy controls (5.0; IQR:1.0-10.0) (z=-9.832; P<0.001), and this is consistent with previous studies. Furthermore, a positive and significant correlation between depressive symptoms and duration of heroin addiction was established, which supports the bidirectional relationship of depression and addiction.

Taken together, our results show for the first time an association of *PDYN* gene variations with the intensity of depressive symptoms and negative craving in heroin addicts. Our findings reveal the association of *PDYN* haplotypes composed of rs2281285, rs2235749 and rs910080 with heroin dependence.

Funding: This study was supported by the Ankara University Scientific Research Projects Coordination Unit (grant number: 20H0217001 awarded to Dilek Kaya-Akyüzlü).

Author contributions: DKA developed the study concept. DKA and SÖK, conducted the genetic analysis and wrote the manuscript. ŞYŞ and NA collected venous blood samples of all subjects.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Kaya-Akyuzlu%20et%20al_1708_Supplementary%20Material.pdf